Urol Res (1995) 23:377–380

ORIGINAL PAPER

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Immortalisation of human urothelial cells

Received: 3 April 1995 / Accepted: 7 July 1995

Abstract A cell line derived from the urothelium lining the ureter of a 12-year-old girl was immortalised using a temperature-sensitive SV40 large T-antigen gene construct, and designated UROtsa. Following immortalisation, UROtsa cells expressed SV40 large T-antigen, but did not acquire characteristics of neoplastic transformation, including growth in soft agar or the development of tumours in nude mice. Metaphase spreads had a normal chromosomal appearance and number. UROtsa cells remained permissive for cell growth at 39 °C, indicating that they did not retain temperature sensitivity. UROtsa provides an in vitro model of "normal" urothelium.

Key words Normal urothelium · In vitro · Immortalisation

Cultures of normal cells grown in vitro go through a limited number of cell divisions before senescing [3]. Immortalisation can occur spontaneously during culture, but it is a rare event in human cells. There are a number of methods for increasing the frequency of immortalisation, including exposure to chemical carcinogens, to transforming viruses or to DNA vectors containing part of the genome of a transforming virus or an oncogene. The frequency with which the transformation takes place varies widely with the origin of the cells and the transforming agent. This paper describes the immortalisation of human urothelial cells using a construct containing a temperature-sensitive variant of the SV40 large T-antigen gene.

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Materials and methods

Primary cultures were produced as previously described [8] and grown in a 3:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% foetal calf serum (FCS), 0.4 μg/ml hydrocortisone (Sigma), 4 μg/ml cholera toxin (ICN-Flow, High Wycombe, UK), 5 μg/ml transferrin (Sigma), 13 ng/ml liothyronine (Koch Light, Suffolk, UK), 24 mg/ml adenine (Sigma) and 20 ng/ml epidermal growth factor (Sigma). The urothelial cells were plated on lethally irradiated Swiss 3T3 feeder cells, as previously described [8].

Transfection

Cells in their second passage were grown to 70% confluence in 25-cm^2 flasks. For transfection, 10 μg pZipU19tsa58 plasmid DNA [4] was added to 1.5 ml Optimem I (Life Technologies, UK) and 30 μl lipofectin (Life Technologies) in 0.5 ml Optimem I. The medium was removed from the cells and the two solutions mixed and added and incubated overnight at 36.5 °C in 8% CO_2. The lipofectin solution was then removed, the cultures washed twice in DMEM and replated with complete medium with feeder cells. The cultures were then incubated at 36.5 °C in an atmosphere of 8% CO_2 and the medium was changed twice weekly. Mock transfections were treated in a similar manner.

Detection of the SV40 large T-antigen

UROtsa cells were grown to 70% confluence on glass slides, fixed in ice-cold methanol/acetone (1:1), washed 3 times with phosphate-buffered saline (PBS) and air dried. The cells were stained with 50 μl undiluted supernatant of murine antibody pAb416 against SV40 large T-antigen (provided by D Lane, University of Dundee) for 1 h at room temperature in a humidified tray. The cells were then washed 3 times with TRIS-buffered saline (TBS) for 5 min each and 50 μl of a 1 in 50 dilution of rabbit anti-mouse Ig antibody conjugated to horseradish peroxidase (Dako) added for 1 h at room

temperature in a humidified tray. The cells were then washed 4 times in PBS for 5 min each. Six milligrams of 3,3″-diamino-benzidine (Sigma) was dissolved in 10 ml TBS, 0.1 ml 3% hydrogen peroxide added and 200 µl of the mixture added to the cells for 10 min. The slides were then rinsed in distilled water, counter-stained with haematoxylin/eosin, and mounted in Urinert (Gurr microscopy materials, BDH).

Karotyping

Cells were grown to approximately 60% confluence in a 25-cm^2 Falcon tissue culture flask and incubated in 0.1 µg/ml colcemid (Life Technologies, Gibco) in complete medium for 3 h. The medium was removed and 5 ml hypotonic buffer [KCl 3 g/l, ethylenediamine-tetra-acetic acid (EDTA) 0.2 g/l, hydroxyethylpiperazine ethanesulphonic acid (HEPES) 4.8 g/l in distilled water, pH 7.4) was added for 20 min. The cells were detached by gentle shaking, centrifuged at 3000 rpm for 5 min and fixed in freshly prepared methanol:glacial acetic acid (3:1) for 30 min. The fixed cells were centrifuged at 1000 rpm for 5 min and resuspended in fresh fixative. Slides were precleaned, cooled to − 20 °C and the cells were dropped onto the slides held at an angle of 30° from a height of approximately 30 cm. The slides were dried at room temperature, rinsed in 70% acetic acid and examined by light microscopy.

Population doubling time

UROtsa cells were plated in 96-well plates in RPMI 1640 medium supplemented with 10% FCS at cell densities ranging from 100 to 3200 in 200 µl medium/well, with eight replicate wells for each cell number. The cells were incubated at 36.5 °C and at 24 h intervals from 3 to 7 days after plating, one plate was removed and 50 µl of a 5 mg/ml aqueous solution of MTT (Sigma) was added to each well, including a row of eight wells lacking cells but containing medium (background control). After a further 3 h incubation at 36.5 °C, all the fluid was removed and 100 µl dimethyl sulphoxide (DMSO) (Sigma) added. The plates were shaken for 5 min at setting 7 on a Rotatest Shaker (Luckham) and read at 540 nm on a Titertek Multiskan MCC/340 plate reader. The background optical densities (ODs) were subtracted from the value for each well, and the average values for the eight replicates for each cell density plotted. The optical density is proportional to cell number. Population doubling time was calculated from the exponential portion of the growth curve using the formula $\log_n 2 \times t/\log_n (OD_t/OD_0)$, where OD_0 and OD_t are the two ODs selected and t is the time interval between.

Growth in soft agar

A lower solid layer of 5% Noble agar (Sigma) in complete medium was poured first into a 5-cm dish, followed by the cells in 0.3% Noble agar. Duplicate gels were set up containing 10^3, 10^4 or 10^5 UROtsa cells, and were incubated at 36.5 °C in 8% CO_2.

Tumourigenicity in athymic mice

Ten million UROtsa cells were suspended in 5 ml PBS and disaggregated by passaging through a 25-g syringe needle. One million cells were injected subcutaneously into the flanks of six nude mice. The mice were kept under a 12-h light/12-h dark regime with free access to food and water. The mice were killed after 6 months and examined for tumours.

Results

A primary culture of urothelial cells derived from the left ureter of a 12-year-old girl was passaged once and transfected with a temperature-sensitive SV40 large T-antigen gene. Eighteen weeks after transfection a single colony grew out and was propagated in the absence of feeder cells and designated UROtsa. Control flasks were maintained for an additional 6 weeks until no cells remained. The preliminary characterisation described here was carried out using cells in their first 15 passages. The cells have been passaged over 30 times, and on this basis appear to have an unlimited life span.

The morphology of the cells closely resembled that of primary cultures of normal human urothelial cells in the Rheinwald-Green medium. The cells grew in tightly packed colonies consisting of irregular polygonal cells with a low nucleus to cytoplasmic ratio (Fig. 1). Multinucleated cells occurred at a low frequency, as did long

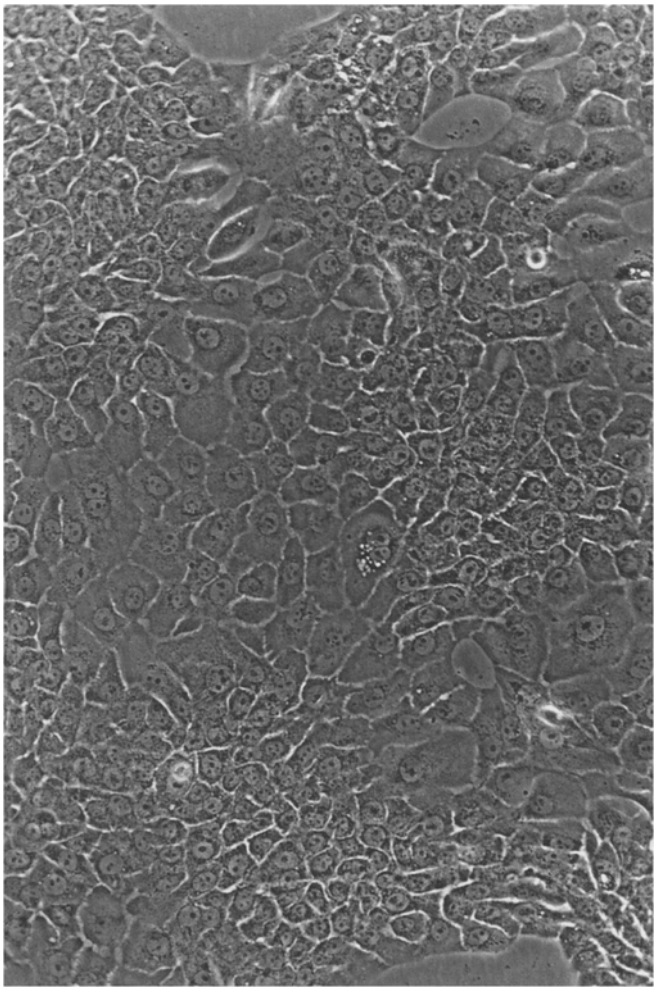

Fig. 1 Photomicrograph of UROtsa cells growing on plastic in RPMI 1640 medium supplemented with 10% FCS. Denser patches of epithelioid cells are interspersed with more flattened cells with an irregular outline. Phase contrast, × 50

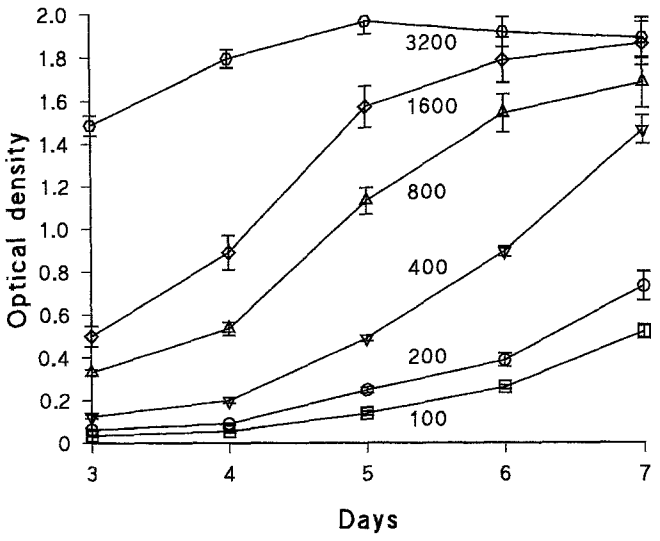

Fig. 2 Growth rates of UROtsa cells in 96-well plates at densities ranging from 100 to 3200/well, 3–7 days after being plated. At low cell densities the cells are still in lag phase 5–6 days after plating. At the highest cell density, 3200/well, the cells are already approaching confluence after 3 days. Population doubling time measurements were made using the growth curves of the intermediate cell densities during their exponential phase of growth. Cell numbers are proportional to the optical densities

fusiform cells. All the cells expressed SV40 large T-antigen. In metaphase spreads the number of chromosomes was consistently 46 and there were 2 X chromosomes in each spread, confirming the female origin. No gross chromosomal abnormalities were observed.

In complete Rheinwald-Green medium the cell number increased tenfold over a 10-day culture period. The mitogens used in the Rheinwald-Green medium [epidermal growth factor (EGF), transferrin, hydrocortisone, liothyronine and cholera toxin] were added individually to Rheinwald-Green medium containing minimal (2%) FCS. Only EGF significantly stimulated cell growth in medium lacking the other mitogens. In RPMI 1640 with 10% FCS the cell number increased threefold over this period.

UROtsa was adapted to grow in RPMI 1640 medium supplemented with 10% FCS and stocks were frozen down. This tissue culture medium is cheaper and simpler to use than the complete Rheinwald-Green formulation. After a lag phase at low cell densities, the cells grew relatively quickly until confluent (Fig. 2). Three independent calculations of population-doubling times were made from exponential regions of the dose-response curves, giving values of 26.0, 29.3 and 33.8 h, with a mean of 29.7 h.

When the temperature was raised to 39 °C, the cells continued to grow at the same rate, suggesting that the SV40 T-antigen had lost temperature sensitivity. When UROtsa was grown in soft agar, isolated viable cells could be seen, but no colonies developed. Following transplantation of UROtsa to athymic nude mice, no tumours developed within 6 months.

Discussion

This paper describes the production and preliminary characterisation of an immortalised cell line derived from normal urothelium. The cells failed to grow in soft agar or following transplantation to nude mice, indicating that they do not have a neoplastically transformed phenotype. The cells have been passaged over 30 times, and on this basis may have an unlimited life span.

The first publication describing the immortalisation of human urothelial cells was from Reznikoff's group [2], who used live wild-type SV40. The resultant cell line, SV-HUC, was similar to the parent cells in morphology, karotype and medium requirements at early passage. Although the cells were non-tumourigenic, they did gain the ability to grow in soft agar. Within 15 passages the cells were found to be cytogenetically unstable [6]. Induction of neoplastic transformation in SV-HUC cells with chemical carcinogens such as 3-methylcholanthrene [10], 4-aminobiphenyl [1] or X-rays [7] or transfection with a mutated *ras* oncogene [9] was associated with losses on chromosome arms 1p, 3p, 4, 8, 10p, 11p, 13q, and 18. There is evidence that these carcinogen-induced tumours of SV-HUC are associated with amplification of SV40 DNA mapped to a common fragile site on 9q12-21.1pter [5]. The similarity between the genetic events leading to the neoplastic progression of a "normal" urothelial cell line in vitro and the common genetic changes seen in bladder cancer biopsies [11] emphasises how valuable such in vitro models can be. Although we failed to observe genetic changes at a gross level in UROtsa, SV40 DNA has been integrated into the genome which, from the studies on SV-HUC, will result in genetic instability and chromosomal changes within 15 passages. It will be of interest to learn where the SV40 DNA is integrated in the UROtsa genome and what additional genetic losses occur.

A progressive loss of differentiated characteristics is observed in most cell lines immortalised with SV40. The aim of our study was to immortalise cells with a temperature-sensitive construct, such that cell proliferation could be halted at the non-permissive temperature. It was hoped in this way to provide an in vitro model system that matched human urothelial cells in vivo more closely than currently available models such as SV-HUC, by providing cells in which the contribution of the viral genome could be switched off. The transfected cells were selected at 36.5 °C, because experience with keratinocytes has shown that selection at 33° C does not result in immortalised cell lines, despite this temperature being fully permissive for the construct. However, the UROtsa cells also continued to

grow progressively at the non-permissive temperature (39 °C), indicating that the T-antigen was no longer temperature sensitive. It is possible that these cells have undergone another genetic change conferring immortality, such that they are independent of SV40 T-antigen expression. For example, the SV40 DNA may have been integrated in a position that activates another gene necessary for continuous cell growth. Despite the lack of temperature sensitivity, the UROtsa cell line closely resembles the parental cells from which it was derived and provides a model system for the study of human "normal" urothelial cells.

Acknowledgement This work was supported by the St. Peter's Research Trust.

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